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Fixation of free Nitrogen By Living Organisms

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THE FIXATION OF FREE NITROGEN BY LIVING ORGANISMS

BY

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THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

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ENTITLED THE FIXATION OF FREE NITROGEN BY LIVING ORGANISMS.

IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE DEGREE

OF BACHELOR OF SCIENCE

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The Fixation of Free Nitrogen by Living Organisms.

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Through what Agents?

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Introduction.

The purpose of this paper is to give a review of the work on nitrogen fixation, and its present status including our own investigations. The points covered by our experiments are the following: the per cent of acidity and of alkalinity in which legume bacteria grow; the fixation of nitrogen in pure cultures of bacteria; cultural characteristics; and cross inoculation of bacteria on legumes, with special attention given to the latter point. We have written up the subject according to the outline given, and have used our own results together with those of others as evidence in support of the various points.

Early Investigations.

Tubercles on various species of Leguminosae and a few other plants have been recognized and used as a means of classification for several centuries. In 1615 Lechamp and DeCandolle found them on the roots of Ornithopus and used them in their classification. Since that time, during the seventeenth, eighteenth, and early part of the ninteenth century we find frequent and short mention of the presence of tubercles and various notions assigned for their cause. Some thought them to be natural organs for absorbing and storing food, and others considered them fungoid growths; but no one seemed to have sufficient interest to investigate their real nature.

It is not until 1866 that we find any successful attempt to solve the true function of the legume tubercle. At that time Woronin described their microscopic structure. He found them to consist of an outer vascular rind and an inner soft cellular tissue. This central tissue he found to contain bodies of various shapes which he regarded as living organisms and to which he gave the name of

bacteroids. In 1874 Erickson found branching mycelial-like threads ramifying through the tubercle. These he took for fungus growths and considered them the cause of the tubercle.

In the investigations of these two men we have the foundation of all the later discoveries upon this subject. Upon these two forms, the bacteroids of Woronin, and the hyphae of Erickson, were built up two principal theories, about which there has been a great deal of dissention and in which most of the investigators of the day took sides. These theories are called the exogenous and endogenous theories. The exogenous theory maintains that the filaments are mycelia of a fungus and that the bacteroids are buds or gonidia thrown off from the latter. This theory has been upheld by Laurent, Atkinson, Frank, and Ward. The endogenous theory as first set forth by Prazmowskie (1889), mantains that the tubercles are caused by a bacterium present in the soil, which forces its way into the young rootlets, multiplies and by irritation causes a multiplication of the plant cells. He adopted the name given by Byerinck, Bacillus *Radicicola*, for the infecting organism. He accounts for the hyphae of Erickson by saying that it is a gelatinous envelope formed by the bacteria for protection against the plant juices. Within this envelope are straight rods, without the plant juices react with the bacteria producing involution or branching forms called bacteroids. Prazmowski's theory is upheld in its main points by Byer~~ni~~inck and Wilfarth, and seems to be favored by later investigators.

Hellriegel (1886) was the first to prove that bacteria work in connection with legumes to form tubercles and to fix free nitrogen. Peas were grown in sand, to which all mineral plant food had been added. Water from soil in which peas had been grown was added, and one

half the pots were sterilized. In the first case no tubercles developed and the plants died. In the second case (unsterilized) numerous tubercles developed and the plants flourished.

In 1888 Byerin~~ni~~ck succeeded in cultivating the legume organism upon artificial media, and in 1890 Prazmowski produced tubercles from such artificial cultures.

This includes all the principal points in the investigations of the legume organism. The present status will be treated more fully under the separate topics following.

II. Is Free Nitrogen Fixed by Higher Plants? Through What Agents?

The experiments of all careful investigators have gone to show that, if any of the higher plants be grown in sterilized sand free from nitrogen to which has been added all the necessary elements of plant food except nitrogen, they grow for a short time and then die. In all such cases analysis shows such plants to contain approximately the same amount of nitrogen as is found in the seed planted. If an amount of nitrogen insufficient to mature the plants be added to such pots, the plants will continue to grow until the supplied nitrogen is utilized and then die as before. Hence we conclude that none of the higher plants fix free nitrogen when grown under sterile conditions.

It is, however, the behavior of plants under natural conditions which is of greatest interest, because of the practical bearing it may have. During the earlier period of investigations there was considerable controversy as to which, if any, of the higher plants fix free nitrogen when not grown under sterile conditions. More recent investigations, however, have proved quite conclusively that, with a few important exceptions, none of the phanerogams fix free nitrogen under any known conditions. The exceptions so far as noted are some plants belonging to the Leguminosae, the Elaeaginaceae, Alnus of the Betulaceae, the Myricaceae, the Scrophulariaceae and probably Isopyrum biternatum, all of which produce tubercles, to a greater or less extent when properly infected, the latter condition also being a requisite to nitrogen fixation.

The Leguminosae, because of their economic importance, have attracted the attention of more investigators then the other plants

mentioned. Their ability to fix free nitrogen, when properly infected, has been proven beyond question by such eminent workers as Hellriegel, Nobbe, H~~e~~iltner, Hotte~~s~~r, Schmidt, Schloesingfi~~t~~ls and Laurent, Lawes and Gilbert, Ward, Atwater and Woods. As examples of the proofs establishing this point, there follow below the results of two experiments from Hellriegel's work and one from that of Schloesingfi~~t~~ls and Laurent. Also see table VII of our own work.

Hellriegel's Experiments.

1. The plants used were grown in a large carboy, under sterile conditions, with a controlled air supply. The nitrogen balance obtained was as follows;-

Combined N. in air originally filling carboy=less than	0.0001	grams
" " " sand used	0.0000	"
" " " nutritive solution and twice distilled water	0.0000	"
" " " soil infusions	0.0002	"
" " " three oat seeds	0.0007	"
" " " three pea seeds	0.0081	"
" " " three buckwheat seeds	0.0004	"
Total nitrogen supplied	0.0095	

Nitrogen found at end of experiment.

In pea plants	0.2335	grams
In oat plants	0.0033	"
In buckwheat plants	0.0006	"
In soil	0.0207	"
Total notrogen found	0.2581	"
Total nitrogen found	0.2581	"
Total nitrogen supplied	0.0095	"
Difference	0.2486	"

2. Experiment performed with Lupinus luteus to show benefit of proper inoculation. Two plants grown per pot.

	Yield of dry matter in grams.	Wt. of N. therein	Wt. of N. Supplied in Seed, soil & water.	gain or loss of N.
I. With tubercle formation.				
(A).	38.919	0.998	0.022	+ 0.975
(B).	33.755	0.981	0.023	+ 0.958
II. Without tubercle formation.				
(C).	0.989	0.016	0.020	- 0.004
(D).	0.828	0.011	0.022	- 0.009

Schloesing fils and Laurent's Experiment.

Inoculated legumes were grown in closed vessels containing sterilized soil. The free nitrogen in the enclosed air was determined at the beginning and close of the experiment with the following results;-

	1	2
Free N. in air (initial)	2681.2 c.c.	2483.3 c.c.
Free N. in air (final)	2652.1 c.c.	2457.4 c.c.
Difference	29.1 c.c.=36.5mgs.	25.9 c.c.=32.5 mgs.

Analysis of the plants grown showed a gain of nitrogen very similar to that lost from the air during the period of their growth. The latter experiment not only proves the fixation of nitrogen but shows conclusively that the source of the nitrogen fixed is the free nitrogen of the air.

Data showing the fixation of nitrogen by the other plants mentioned are much less abundant. Nobbe, Schmidt, Hiltner and Hotter found that Elaeagnus plants, the roots of which develop tubercles due to the invasion of a fungus totally different from the one causing the leguminous nodules, also fix and assimilate the free nitrogen of the atmosphere. This was shown by the fact that the infected plants

grew much more rapidly and vigorously than similar uninfected plants grown side by side with them.

Hiltner proved that the alder (Alnus), when inoculated produced tubercles and fixed free nitrogen. When grown under sterile conditions, however, it failed to do either. The present status of investigations seems to indicate a close correlation between tubercle production and nitrogen fixation.

The fixation of atmospheric nitrogen having been established naturally the next question to be taken up by investigators was the cause of such fixation. Hellriegel, early in his investigations, proved that it was due to some living organism in the soil. Four pots of lupines were grown under sterile conditions. Similar lupine soil infusions were added to all the pots, those added to the first two, however, having first been boiled, with the following results.

Pot No.	Treatment.	Dry matter in grams.	N. acquired from air in grams.
1.	Inoculated with sterilized soil infusion	0.926	-0.007
2.	" " " " "	1.008	-0.007
3.	" " soil infusion	42.681	+1.147
4.	" " " "	40.574	+1.054

Later he succeeded in producing abundant tubercles with accompaning nitrogen fixation by inoculating plants from tubercles produced upon other plants of the same kind; Also by dipping plants in water containing pulverized tubercles, while in both instances check plants showed neither result. These results, confirmed by Beyerinck, Prazmowski, Ward and others, seemed to point to some living organism developing in the tubercle as the primary source of infection.

It was not long till Beyerinck succeeded in isolating a bacillus from the tubercles which he proved, by inoculation experiments, to be the active agent in tubercle production. He named this organism Bacillus radicicola, which name has met with quite general acceptance. Prazmowski was the first to confirm Beyerinck's work. By inoculating plants with pure cultures of this bacillus he secured the following results.

Pot No.	Treatment.	Dry matter in crop g.	g.N.in crop.	g.N.in seed.
1.	With Na NO_3	3.5492	0.0892	0.0090
2.	" " and bacilli	5.3280	0.1579	0.0090
3.	Without Na NO_3	0.4124	0.0072	0.0090
4.	" " and with bacilli	2.4755	0.0583	0.0090

Thus it has been proven that Bacillus radicicola is the causal agent in tubercle production and nitrogen fixation in legumes, that it develops in tubercles, and that it exists in soils upon which infected plants have been grown.

III. Development of the Tubercle.

Having determined the agent of tubercle production, the manner of its infection and the nature of the resulting growth were next taken up. By careful microscopical study of tubercles in the different stages of their development, Prazmowski and later Frank, Laurent, Ward and Atkinson succeeded in following the various physiological changes involved in their growth.

The Bacillus radicicola, existing in the soil or applied by inoculation, comes in contact with the fine hairs abounding upon the growing rootlets. One or more, probably by the secretion of an enzyme which dissolves the cell wall, enters a root-hair and there, nourished by the nutritive plant juices, multiplies rapidly forming a colony. This colony, in most cases, soon envelopes itself in a thin membrane, presumably for protection against some injurious action of the plant plasma. The presence of this protecting membrane, resembling mycelial threads, misled many of the earlier investigators regarding the nature of the infecting organism, they mistaking these infecting threads for the hyphae of some low fungus. The enclosing membrane is rarely found in the tubercles of lupines. Upon reaching the cortical parenchyma the infecting thread branches freely passing by a flexuous course through and between the cells to the endodermis tissue, until those cells near the point of infection are thoroughly permeated by the branching filament. The enclosing membrane seems to become quite small or constricted in passing through the cell walls, expanding greatly within the cells, thus presenting a nodulose appearance. The openings in the cell walls are probably produced by the secretion of an enzyme capable of dissolving the cellulose, in the same manner that the first entrance into the root hair is

effected. The presence of the invading organism results in a violent stimulation in the growth of the infected tissue, producing an abnormal growth known as the tubercle. After a time the membrane enclosing the infecting colony, due either to a loss of vigor on the part of the bacteria or to an increase in the aggressiveness of the plant's growth forces, is ruptured and the bacteria set free in the plant cells. The organisms, thus exposed to the action of the plant plasma, cease to divide and soon assume irregular club shaped or branching forms known as involution forms. This change, evidently, is an intermediate step in their assimilation by the plant. After acquiring the involution form the bacteria soon begin to disappear. The co-incidence of this change with an increased vigor of the plant seems to indicate that the protoplasm of the bacterial cell thus absorbed is utilized by the plant in its growth. The assimilation process begins near the basal or older portion of the tubercle and gradually extends as the plant nears maturity until usually almost the entire contents of the tubercle have been absorbed. A portion of the bacteria in the growing part of the tubercle, varying with the condition, generally escape destruction and upon the decay of the enveloping tissue are set free in the soil.

Most modern investigators agree in the main with the development of the tubercle as outlined above. Frank, however, maintains that the membrane enveloping the infecting colony may be a product of the plant rather than of the bacteria. A.Koch and M.W.Beyerinck found that it was stained blue by zinc iodochloride which would show that it was composed of a substance allied to cellulose. The formation of bacteroids, though a general stage, is not necessarily a constant one in the development of the bacteria within the tubercle.

Its presence seems to depend upon the comparative virulence of the bacteria and vigor of the plant. In the absence of the bacteroidal stage, the plant seems to get no benefit from the presence of the infecting organism, in other words it becomes a parasite. Evidence goes to show that in some cases the Bacillus radicicola may benefit the host plant by developing in the root cells without the production of tubercles.

IV. Where and How is Nitrogen Fixed.

Three theories have been advanced as solving the problem of where and how nitrogen is fixed. The first one holds that gaseous nitrogen is taken up directly by the leaves of the plant just as is carbon dioxide gas. Frank, who was the leading advocate of this theory, maintained that not only legumes but other plants, to a greater or less extent, took up free nitrogen through their leaves and utilized it in their growth. Numerous experiments, however, have proven this view entirely untenable.

The second and more generally accepted theory maintains that gaseous nitrogen is fixed by the legume and bacteria working together symbiotically. This theory leaves the exact process of fixation a mystery. Kossowitch, performed an experiment for the purpose of locating as nearly as possible the place of fixation. Nitrogen was excluded alternately from the roots and tops of growing plants, the air being replaced by hydrogen and oxygen gas. Though the plants were somewhat injured by the conditions of the experiment, yet the results seemed to show quite conclusively that only the roots take up free nitrogen. Further, the analysis of tubercles and the remainder of plants separately show the former to contain from seven to eight per

cent of nitrogen and the latter but two per cent. This would seem to indicate that the seat of nitrogen fixation is not only in the root but in the nodulose growths of the root. Recent investigations, however, especially those of Nobbe and Hiltner confirmed by others showing the possibility under certain conditions of abundant infection and tubercle production without benefit to the host plant, makes it impossible longer to accept the theory of Symbiosis.

The latest, and now quite generally accepted view, locates the power of nitrogen fixation in the bacteria, the plant simply utilizing the nitrogen so fixed. This theory has seemingly been established beyond question by the fact that <u>Bacillus radicicola</u> has been proven, by Berthelot, Laurent, Moore, Maze, ourselves and probably others, to possess the faculty of fixing free nitrogen when grown in artificial cultures. Though we accept this view there still remains the question as to whether the plant secures its nitrogen by a direct assimilation of the bacteroidal protoplasm or of some nitrogenous excretion product of the bacteria. In order to decide this point, Dr G.Moore of the U.S. Department of Agriculture grew legume bacteria in one hundred cubic centimeters of nitrogen-free nutrient solution. After the elapse of a period previously determined to be sufficient for the accumulation of a readily determinable fixation, the bacteria were screened from the solution by passing it through a Pasteur-Chamberland filter. The filtrate when analyzed showed a trace of nitrogen, but principally all the fixed nitrogen had been appropriated for the growth of the organisms themselves. Hence, according to the present view the bacillus is a parasite, having the power of fixing free nitrogen and utilizing it in the growth of its own cells. The infected legume plants secure their nitrogen by overcoming the

invading organism and then assimilating the protoplasmic contents of the resulting bacteroidal cells.

V. Do Lower Forms of Plant Life Fix Free Nitrogen?

There have been great accumulations of nitrogenous matter in many places on the surface of the earth where no legumes, or in fact no higher plants have ever grown. On almost bare rock we find bacteria, algae, and lichens, growing and doing well where there is practically no combined nitrogen. We must, in some way, account for such fixations; and we can only do this by ascribing a part of it, at least, to the lower forms of plant life.

There have been numerous experiments made to show that nitrogen is fixed in the soil and in cultures by micro-organisms. Berthelot has done a great deal along this line, using bacteria, algae, and higher fungi in his experiments. Some of his results are given in the following tables:-

Table showing the fixation of nitrogen by mixed cultures of soil bacteria in 3½ Mo.

Nutritive media.	Size of Flask	Nitrogen Initial	Final	% gained
Humic acid	1 liter	7.7	12.2	57
" "	6 "	38.4	36.	
Humic acid and Kaolin	1 "	8.3	12.7	52
Kaolin	1 "	7.9	19.7	150
Cohn's solution	6 "	13.3	19.2	44
Cohn's sol. (check)	6 "	13.	14.	

Table showing nitrogen fixation by Aspergillus Niger in one Month.

Nutritive media.	Size of Flask	Nitrogen Initial	Final	% gained
Cohn's sol.(check)	6 liter	24.9	24.4	--
" "	6 "	24.9	31.3	26

Table showing nitrogen fixation by algae.

Nutritive media.	Culture	Nitrogen Initial	Final	% gained
5 cc water 5 gr.humic acid	2 cc water containing algae	.1805	.1909	6
100 cc water 5 gr.humic acid	2 cc water containing algae	.1805	.1961	9
5 " " "	air from soil	.1805	.2350	30.3
5 " " "	check	.1805	.1805	____

In the above experiments we find a considerable fixation of nitrogen in all cases, excepting the checks and the six liter flask of soil bacteria, found in the first table. Large bodies of media do not seem to give as good results as smaller ones; a fact which is probably due to an insufficient supply of air. In other experiments he found that by isolating the bacteria and algae used in those experiments, only part of them were capable of fixing free nitrogen. It will be observed that the culture media used above are, in most cases, soil products. In his experiments with soil he was able to get from 75--100# nitrogen fixed per acre and in a few cases much more.

Laurent, also, worked with algae. His experiment differs from Berthelot's in that he used known forms of organisms and made both a direct and indirect determination of the nitrogen fixed. (The direct being obtained by measuring nitrogen in the air at the beginning and

end and the indirect from an analysis of the plants.)

Nutrient Media	Culture	Time of exp.	Direct determination of N mgs Initial	mgs final	mgs loss from air	Indirect determination of N mgs Initial	mgs final	mgs gain
600 grams sub-soil	Nostoc punctiforme " Minutum	6 mo	1208.37	1145.37	+63.	73.5	136.1	62.6
600 grams sub-soil	Nostoc punctiforme " Minutum	"	1042.01	1004.91	+37.1	73.5	114.8	41.3
600 grams quartz sand	Nostoc punctiforme	5 mo	1288.694	1251.894	+36.8	1.	36.2	35.2
600 grams quartz sand	" "	"				1.	34.	33.
600 grams sub-soil	Brachythecium rutabulum Barbula muralis	3 mo	1315.46	1315.362	+ .1	68.4	68.	-.4
600 grams sub-soil	Micrococcus vaginalus	"	964.935	965.335	-.4	66.4	66.5	.1

In the first four cultures there was considerable fixation as shown both by the direct and the indirect determinations. In the two mosses and the Oscillaria there are no evidences of such fixation.

Petermann worked with sterilized and unsterilized soil with the following results;

Sterilized	Unsterilized
1. 1.7 Mgs N loss	1. 3.9 Mgs N gain
2. .8 Mgs N loss	2. 3.1 Mgs N gain

Nobbe and Hiltner performed an experiment for the purpose of showing the relation existing between the crop grown and the amount of nitrogen fixed in the soil. They made a soil from a mixture of sand and loam, to which they added mineral fertilizers and mixed so that it would have a uniform nitrogen content. This soil they divided in 5 sets of jars, which they sterilized and planted to the following crops: peas, mustard, buckwheat, and oats. The fifth set was not planted.

	Peas	Mustard	Buckwheat	Oats	Bare soil
Grams N in soil (initial)	3.320	3.320	3.320	3.320	3.320
Grams N in seed	.401	.018	.027	.048	
Sum of N in soil and seed	3.721	3.338	3.347	3.368	3.320
Grams N in tops	.684	.237	.234	.261	
Grams N in roots	.175	.068	.042	.226	
Grams N in soil (final)	3.399	3.269	3.326	3.618	3.374
Sum of N in plant and soil	4.258	3.574	3.602	4.105	3.374
Grams N gained	.537	.236	.255	.737	.054

From an examination of this table it is evident; that nitrogen was fixed in all the jars, even in the bare soil; that more nitrogen was fixed where a crop was grown than in the bare soil; that in the pea jars the greater per cent of nitrogen fixed appears in the crop while in the other cases the fixation does not greatly benefit the crop; that there was more nitrogen fixed where oats were grown than where peas were grown. The next year another crop was grown and it was found that the nitrogen fixed in the soil had become available for crop production. The conclusion to be drawn from these results, is that some higher plants enable micro-organisms to grow and fix nitrogen in the soil; but that such nitrogen is not immediately available to the plant.

Winogradsky was the first to separate from the soil a bacterium which would fix free nitrogen in pure culture. He named this bacterium Cloistridium Pasteuranium. He found that it was capable of fixing from 2.5 to 3 parts nitrogen per thousand parts media. This organism was an anaerobie which seemed to do its best work when grown with aerobic forms with a small amount of air present. Since this discovery several other species having the same power have been isolated by German investigators and have been put on the market in pure cultures under the name, Alinit. This substance was advertised as greatly increasing crop yields and for a time had a considerable sale. In many cases it gave excellent results, but the per cent of failures was so high that the practice was finally abandoned.

Again, there is considerable evidence that the legume bacteria are capable of fixing free nitrogen in the soil outside the plant. In the first place this is shown by the fact that they will grow well in nitrogen-free media where many other species of bacteria

will not grow; and as their bodies are proteid in nature they must obtain nitrogen from the atmosphere. In the second place, direct determinations of nitrogen in media where legume bacteria have been grown, have been made to show such fixation. Beyerinck got a fixation of 18 mgs.nitrogen per liter of media in three months time. Moore of the Department of Agriculture got from .2-32 mgs. nitrogen per 100 c.c. in ninety different tests; and in another test with different media he obtained as high as 3.1 mgs. nitrogen per 100 c.c. Our own results on this subject are given below;-

Table I.

Nitrogen Fixed in 300 c.c.of Media in 1 month.

Bacterium	Mgs.total N in Media	Mgs. N Fixed.
1. Check	1.17	0
2. Check	1.12	0
Average of Checks	1.145	0
3. Red clover	1.240	.095
4. White clover	1.240	.095
5. Alsike "	1.688	.543
6. Alsike "	1.800	.655
7. Crimson "	1.460	.315
8. Crimson "	2.137	.992
9. Alfalfa	1.688	.543
10.Alfalfa	1.460	.315
11.Sweet clover	1.240	.095
12 Sweet "	1.290	.145
13.Soy bean	1.460	.315
14.Soy "	1.245	,095

From the above table it appears that in every case where there was inoculation there was a small fixation of nitrogen. The differences shown between cultures of the same organisms are readily to be accounted for by differences in the growth of the cultures, for while they were all made in the same media there are always inequalities of growth. From the above results we conclude that algae, bacteria and other lower forms of plant life do fix free nitrogen in their bodies and finally contribute this to the soil in amounts which may be of considerable importance to agriculture.

VI. A Study of the Relationships Existing Among the Bacteria of Different Legumes.

(A) Legumes Selected for Experiments.

There are hundreds of species of legumes and other plants bearing tubercles, as it was impossible for us to use many of these, we selected for our experiment fifteen species of legumes which are widely grown and which are of considerable economic importance. The plants selected were the following:-

Common name--	Family	Species
1.Red clover	Papilionaceae	*Trifolium pratense*
2.White "	"	" *repens*
3.Alsike "	"	" *hybridium*
4.Crimson "	"	" *incarnatum*
5.Alfalfa	"	*Medicago sativia*
6.Sweet clover	"	*Melilotus alba*
7.Cow pea	"	*Vigna Catiang*
8.Soy bean	"	*Glycine hispda*
9.Garden bean	"	*Phaseolus vulgaris*
10.	"	*Apios tuberosa*
11.Garden pea	"	*Pisum sativum*
12.Sweet pea	"	*Lathyrus odoratus*
13.Vetch	"	*Vicia sativa*
14.Canada Field pea	"	*Pisum sativum*
15.Partridge pea	Caesalpineaceae	*Cassia Chamaecrista*

(B) Size, Form and Nature of Bacteria in Tubercles.

Upon examining the tubercles from any one species of legume, we found but small variations in the form and size of bacteria taken from tubercles of different ages. Again, by comparing the bacteria taken from tubercles of different species we found in many cases wide and constant variations which enabled us to distinguish the organism from those of other species of legumes. In other cases they were so nearly alike that we could observe no difference. The following sketches will give an idea of the comparative sizes and forms of the bacteroids taken from the legumes we have studied. These sketches represent a magnification of 5000 diameters.

The sizes were determined by the use of the micrometer and drawn to scale.

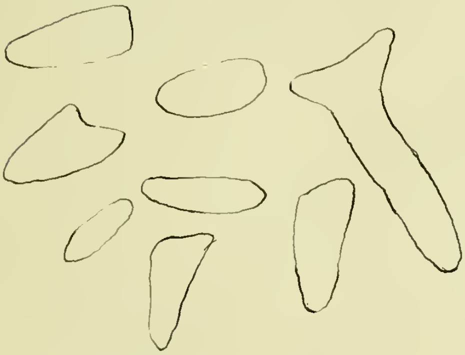
Group I.

Red clover

Width .7-2 μ

Length 2.1-5.2 μ

White clover



Width .7-2 μ

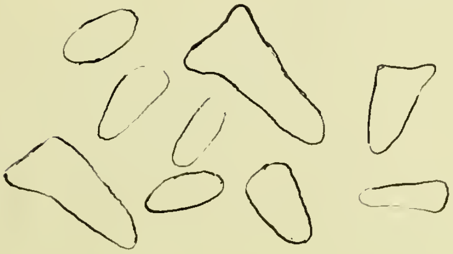
Length 3.6 μ

Alsike clover

Width .7-2 μ

Length 2.1-6 μ

Crimson clover

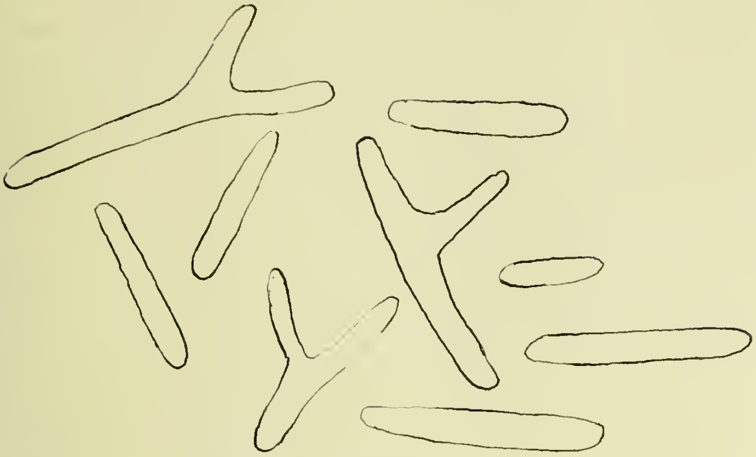


Width .7-2 μ

Length 1.9-4.2 μ

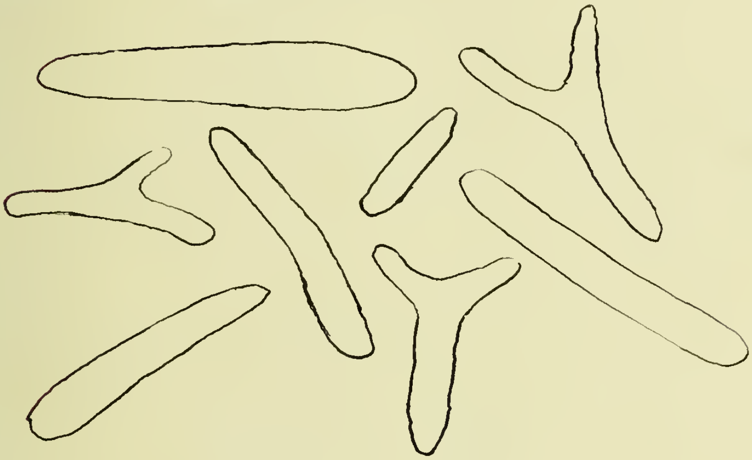
Group II.

Alfalfa



Width	.7+	μ
Length	2.2-8.4	μ

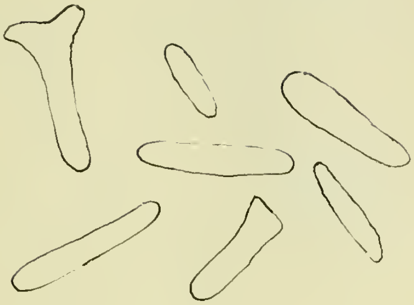
Sweet clover



Width	.8+	μ
Length	3-9	μ

Group III.

Cow pea



Width .5+ μ

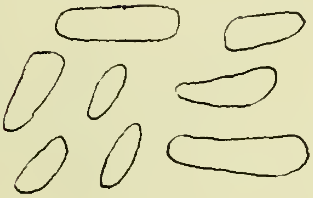
Length 2-4-2 μ

Soy bean

Width .5+ μ

Length 2-4 μ

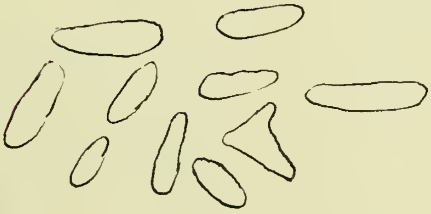
Garden bean



Width .7 μ

Length 1.1-3.4 μ

Apios

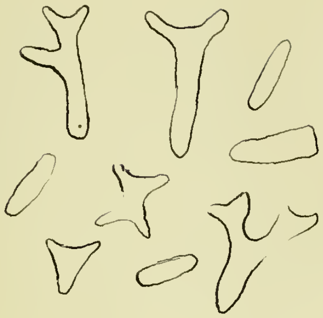


Width .5+ μ

Length 1. -2.8 μ

Group IV.

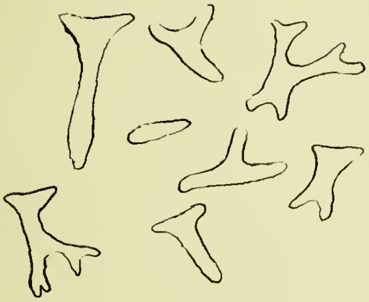
Garden pea



Width .5-.7 μ

Length 1-4.2 μ

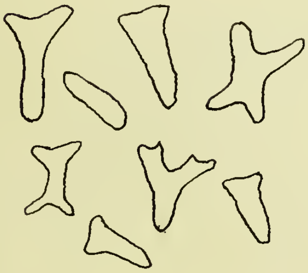
Vetch



Width .5-.7 μ

Length 1- 4 μ

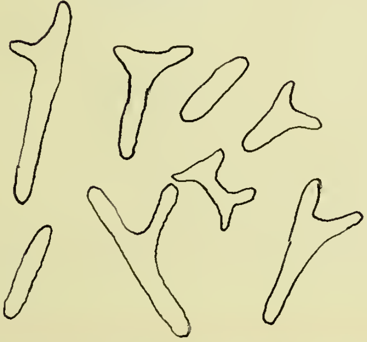
Sweet pea



Width .5- 1 μ

Length 1- 3.5 μ

Cassia



Width .5 μ

Length 1.5-5. μ

The above sketches show that there are four general types of bacteria in the fourteen plants represented. We were unable to secure Canada field pea tubercles, hence it is not included here.

In the first group are the four clovers, red, white, alsike, and crimson. These all have bacteria very much alike, both in size and in form. There are very few branched forms but many wedge shapes, and rods. The crimson clover bacteri~~a~~um is slightly different from the others in that it is shorter and thicker.

In the second group are included alfalfa and sweet clover, both of which are readily differenciated from the other groups by their large sized bacteria. These two forms are very much alike and in fact only differ in that the alfalfa is not quite so wide as the sweet clover bacteria.

The third group, soy bean, cow pea, garden bean, and Apios are associated together because of their small size and their tendency to produce only rod forms. The Apios bacteria is smaller than the other three.

Garden pea, sweet pea, vetch, partridge pea and Canada field pea compose the fourth group. The bacteria in this group are very similar in that they are near the same size and have widely branching forms. The first three kinds are so near alike that we could observe practically no difference. The fourth (partridge pea) is a little larger and a little less branched.

Theories as to the cause of the variations between these groups have already been suggested in the foregoing topics; and some proof as to whether such variations represent actual and permanent differences in the organism will be given in our experiments on cross inoculation.

From the sketches given it may be observed that there are both rods and branching forms of bacteria in the tubercle. Byerinck adds to these two, a third form which he calls rovers. These rovers he describes as being .9 μ long, .18 μ wide, and as being actively motile. He further states that the large branching forms are motile as shown by the fact that they wander towards the edge of the drop, when examined in drop culture. We have made careful examination of the bacteria from many tubercles and have failed to find anything corrosponding to these rovers. In drop cultures we have observed the movement of the bacteroids towards the edge of the drop, but beleive this movement to be due to currents set up by evaporation from the edge of the drop. This movement is very slow and steady, and seems to act upon all individuals alike. Hence it seems very improbable that this movement is due to the motility of the bacteria.

Another thing which points toward the non-motility of the legume organism is the fact that these organisms are very slightly diffusable through the soil. Nobbe and Hiltner carried on an experiment which brings out this point very clearly. They first showed that when soils were inoculated only on the surface, they developed tubercles only on the surface roots of legumes. They then tried numerous experiments by introducing the organism at various depths in sterilized soils, and found that in every case infection took place only near the point of inoculation. Further, it seems to be an established fact that heavy soils permit of less diffusion of the bacteria than do sandy soils, because they act as a filter for the bacteria. In the laboratory one can filter out the bacteria from a culture and it seems reasonable, as facts tend to prove, that the soil should also act as a filter.

Assuming this to be true we may conclude that the only efficient inoculation is that which so distributes the bacteria that they will come in contact with the most roots of the plants.

(C) Methods of Securing Pure Cultures of *Bacillus radicicola*.

Fresh tubercles were taken from each kind of legume from which it was desired to secure a culture. The best results were obtained by selecting those of medium size. The very small ones were found to be more difficult to handle and the larger ones were tougher and hence harder to puncture as well as being less juicy in the interior. The tubercles selected were washed thoroughly in distilled water and sterilized for ten minutes in corrosive sublimate (1:1000). They were then taken up in a pair of sterilized tweezers, rinsed thoroughly in sterile water and then in alcohol and passed quickly through a gas flame to burn off the latter. The tubercle now thoroughly sterile was still held in the tweezers and pierced by a sterilized platnium wire. Within the growing tubercle was a light colored, slightly sticky substance some of which adhered to the needle as it was withdrawn. With the needle thus loaded with bacteria the desired medium was inoculated. The time of growth and the percentages of pure cultures secured by us upon chemical agar (for the composition of which see next topic) are shown in the following table.

Table II.

No.	No. of Tubes	Name of host Plant.	Number of tubes showing growth. 1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	8th day	total	% of growth
1.	12	Cow Pea				3	1	2	1	1	8	36
2.	12	Soy Bean			3	2	1				6	50
3.	12	Red Clover			2	4	1				7	58
4.	12	Sweet Clover		2	2	3					7	58
5.	12	White Clover			1	3	2		1		7	58
6.	12	Garden Bean			1	2	1				4	33
7.	12	Alfalfa			1	2	1				4	33
8.	12	Alsike Clover		3	4						7	58
9.	12	Vetch		1	3	2	1	1	1		9	75
10.	18	Partridge Pea		3	3	1		1			8	66
11.	18	Apios			2	2	2	1	1		8	44
12.	18	Garden Pea		4	8	2	2				16	88
13.	18	Crimson Clover		3	7	8					18	100
14.	12	Sweet Pea		1	5	3					9	66

Thus we see that with good media and favorable conditions, growth may appear the second day but may not appear until the seventh or eighth day. Our rather low percentage of growths is accounted for by the fact that the medium used for most of the inoculations was later found to be too acid for the best results. Also the lateness of the season (end of September) made it impossible, in some cases, to secure tubercles in the most favorable condition for making inoculations.

(D). Cultural Characteristics.

The differences in the character of the growths of the bacteria from the different legumes as studied on the different media were very slight. When growing rapidly, they all showed a greater or less degree of viscosity. This trait, however, was a much more marked and constant characteristic of the growth of the clover bacteria than of that of the others. Vetch, soy bean, cow pea and apios produced a growth containing a slightlygreater amount of white pigment than the others, giving it a slightly less transparent appearance. Vetch bacteria seemed to be the most vigorous growers of all. They grew faster and produced heavier growths upon all the media tried.

In comparing the adaptibility of various media for the growth of <u>Bacillus radicicola</u>, six kinds were tested with results as stated below. The solid media all produced similar growths, the differences noted being in the rate and quantity rather than in the character of the growth. The growth made its appearance as small, semi-transparent colonies along the line of inoculation. These, however, because of their fluid nature, soon fused, producing, as the growth increased, a rather narrow, semi-transparent streamer down

the slant, readily distinguishable from any contaminations which might appear. The growth was slightly raised and, when growing rapidly, of a viscous, stringy nature. The distinguishing characteristics of the growths upon the different media will be noted under each.

Plain agar and gelatin. It was difficult to secure growths upon plain agar and gelatin direct from the tubercle. What few grew did so only after the elapse of seven or eight days. After the bacteria had been grown for some time upon artificial media, however, it became comparatively easy to secure such growths. Sluggishness in the rate of growth was the most notable feature of the bacterial growth upon these media.

Chemical and asparagin agar. The chemical agar is composed of 500 c.c. of water, .5 g of $K_2 H P O_4$, .5 g of $Na_2 SO_4$, .375 g of $Mg SO_4$, .1 g of $Ca\ cl_2$, 5 g. of sugar and 7.5 g of agar and the asparagin agar of 500 c.c. of water, 7.5 g of agar, 5 g. of sugar and 1.75 g of asparagin. These media both produced rapid, vigorous growths. We were unable to discern any difference between then either as to rate, quantity, or character of growth.

Liquid media. The liquid medium which produced the best results was composed of 500 c.c. of water, .5 g of $K_2 H P O_4$, .5 g of $Na_2 SO_4$, .375 g of $Mg SO_4$, .1 g of $Ca\ cl_2$, and 5 g of sugar. This medium produced vigorous, rapid growths. These, however, could be distinguished from contamination only by reinoculating back upon the solid medium. The liquid, clear before inoculation would after inoculation gradually become~~s~~ clouded by a whitish growth, finally taking on a milky appearamce. Leaving out the $Ca\ cl_2$ or $Na_2 SO_4$ or both seemed to impair the medium.

All media used was made up to an acidity of from .2 to .3 % Normal.

In making microscopical studies of the bacteria from the different media, occasional involution forms were found. Some authors state that a slightly acid medium is especially favorable to the development of these forms while others maintain that they occur abundantly upon gelatin. We, however, were unable to discover any correlation between their presence and any special characteristic of the medium upon which they were grown. Though we examined numerous drop cultures from the cultural media we found no movements which would indicate motility. Our observations showed that *Bacillus radicicola* grows best in total darkness at a temperature of about 25° C. Strong light retards its growth quite materially though it grows very well in diffused light.

In order to ascertain what per cent acidity or alkalinity of media would produce the maximum growth as well as the range of growth, we made up a series of twenty-four tubes for each legume tested. These tubes ranged from neutral to an acidity of 1.2 % normal, and to alkalinity of 1.1 % normal. They were carefully inoculated and allowed to stand three weeks with results as indicated in tables III and V below. The percentages were made up according to phenothalein indicator, the neutral point of which is about $\frac{N}{.9}$ % acid to lacnoid indicator.

Table III.

Acid Tests. H cl.

Kind of bacteria	$\frac{N}{.1\%}$	$\frac{N}{.2\%}$	$\frac{N}{.3\%}$	$\frac{N}{.4\%}$	$\frac{N}{.5\%}$	$\frac{N}{.6\%}$	$\frac{N}{.7\%}$	$\frac{N}{.8\%}$	$\frac{N}{.9\%}$	$\frac{N}{1.\ \%}$	$\frac{N}{1.1\%}$	$\frac{N}{1.2\%}$
Red clover	vigorous	good	vigorous	good	good	good	good	good	good	0	0	0
White clover	vigorous	good	vigorous	vigorous	vigorous	good	good	good	fair	0	0	0
Alsike clover	good	good	good	good	good	good	good	good	good	0	0	0
Crimson clover	good	good	good	good	good	good	good	fair	fair	0	0	0
Sweet clover	vigorous	vigorous	good	good	good	fair	trace	0	0	0	0	0
Alfalfa	good	vigorous	good	good	good	good	fair	trace	0	0	0	0
Cow pea	vigorous	good	good	good	good	good	fair	fair	fair	0	0	0
Soy Bean	good	vigorous	vigorous	good	good	good	fair	fair	fair	0	0	0
Garden Bean	vigorous	vigorous	vigorous	good	good	good	good	good	good	0	0	0
Garden pea	good	vigorous	vigorous	good	good	good	good	fair	fair	0	0	0
Vetch	vigorous	vigorous	vigorous	vigorous	good	good	good	good	good	0	0	0

The final results, as indicated in the above table, show but slight variations in the growth from $\frac{N}{.1\%}$ to $\frac{N}{.6\%}$. There was, however, a marked variation in the time of the appearance of these growths. The smaller percentages, especially $\frac{N}{.1}$, $\frac{N}{.2}$ and $\frac{N}{.3\%}$, showed quite vigorous growths before the higher percentages showed any, thus indicating that they were more favorable for the growth of the bacillus. Evidently a certain amount of acclimitization had to take place in

the more strongly acid solution, which of course required time.

The results of the test of the different percentages of alkalinity of media follow in table V.

Table V.

Alkali Tests. (Na OH).

Kinds of Bacteria	Neutral	N .1%	N .2%	N .3%	N .4%	N .5%	N .6%	N .7%	N .8%	N .9%	N 1.0%	N 1.1%
Red clover	vigorous	vigorous	good	good	0	0	0	0	0	0	0	0
White clover	"	"	good	good	0	0	0	0	0	0	0	0
Alsike clover	"	"	vigorous	good	0	0	0	0	0	0	0	0
Crimson clover	"	"	good	trace	0	0	0	0	0	0	0	0
Sweet clover	"	"	good	fair	fair	trace	0	0	0	0	0	0
Alfalfa.	"	good	good	fair	trace	0	0	0	0	0	0	0
Cow pea	"	good	good	trace	0	0	0	0	0	0	0	0
Soy bean	"	vigorous	good	fair	0	0	0	0	0	0	0	0
Garden bean	"	good	fair	0	0	0	0	0	0	0	0	0
Vetch	"	vigorous	vigorous	good	good	fair	0	0	0	0	0	0

From this table it is seen that Bacillus radicicola did not withstand so high a per cent of alkalinity as it did of acidity. (phenothalein indicator). The vetch bacteria, as in the acid test, proved to be the most vigorous growers. The sweet clover bacteria which were most strongly affected by the acid, withstood a higher per cent of alkalinity than any excepting the vetch bacteria.

The others showed no very notable differences.

(E). Cross Inoculation.

The inoculation of the bacteria of one legume upon other legumes is a problem which has been worked upon a great deal but the data upon the subject are rather conflicting in nature. Because of this fact and because of the practical value which definite knowledge upon this subject might have, we undertook, so far as possible, to find the result of all such cross inoculations among the fifteen species of legumes we selected for our experiment.

It was considered impractical to grow each plant studied, inoculated with bacteria from all the other species; so in order to get those plants inoculated which would be most likely to show positive results of such inoculation, we resorted to the grouping of the legumes into four groups. In these groups each species of legume was inoculated with every kind of bacterium in the group, excepting its own. We were unable to secure Canada field pea tubercles, and did not see fit to grow the plants Cassia and Apios, consequently, there were a few breaks in the groups. We arrived at a method of grouping after considering botanical relationships of the plants, ~~and~~ similarity in shape and size of tubercle and shape and size of bacteria. The groups are as follows:- Group I. red, white, alsike and crimson clovers. Group II. alfalfa and sweet clover. Group III. cow pea, soy bean, garden bean, Apios. Group IV. garden pea, sweet pea, vetch, partridge pea, Canada field pea.

The style of pot used for growing the plants is that given in the photograph below:-

It consisted of a large glass bell jar 18 inches high and 7.5 inches in diameter at the base. This bell jar was of

sufficient size so that when placed over the gallon jar beneath there was left an opening of about one half inch between them, in which was placed a strip of cotton wool. At the top of the bell jar was an opening one and one half inches in diameter in which was placed a small glass tube wrapped in cotton so as to close the opening in the jar. This tube extended down to the soil beneath and was stoppered above with a cotton plug. The gallon jar beneath was provided with a glass-wool filter for drainage, and was filled with pure white sand. This entire apparatus before setting up was thoroughly sterilized: the glassware by means of a (1: 1000) solution of corrosive sublimate; the cotton and tubes by dry heat for 1 hour at 150° C, the sand by heat for 6 hours at 175° C+. The seeds were sterilized in a (1: 1000) solution of corrosive sublimate. The entire work of sterilization and planting was done in a separate room under as near sterile conditions as possible. The inoculations were made at the time of planting, after which the jars were immediately closed and taken to the green house. As soon as the seed germinated plant food was added in liquid form through the glass tubes. The plant food consisted of all the essential elements excepting nitrogen and was added twice during the period of growth. Owing to the fact that the pots were closed the air could circulate only through the cotton and evaporation was so slow that the plants needed watering only once a month. All water and plant food added were carefully sterilized.

In the following tables the method of inoculating shown is such that each plant is inoculated with bacteria from every other plant in the group; thus red clover is inoculated with white clover bacteria and white clover is inoculated with red clover bacteria. This gives a check on the work, as where infection occurs in both

cases it is positive proof of their ability to cross. The analyses given in table VII represent an average of the analyses of all the plants in the pot calculated to the number given, for convenience in comparing results.

Table VI.

First Series--Closed Pots--(Nov.11, 1904--Jan.28, 1905)

Plant	Inoculated with Bacteria.	Nov.29 In. high	Nov.29 color	Dec.5 In. high	Dec.5 color	Jan.28 In. high	Jan.28 color	Tubercles.
1 Red clover	white clover	1.5	poor	2-3	medium	3	good	numerous
2 " "	alsike "	1.2	"	1.5	poor	3	"	"
3 " "	crimson "	1.	"	1.5	"	2.5	"	"
4 White clover	red "	1.	"	1-2	"	3	medium	"
5 " "	alsike "	1.	"	1-2	"	3	good	"
6 " "	crimson "	1.	"	1-2	"	3	"	"
7 Alsike "	red "	1-1.5	"	1.5-2	medium	2	medium	"
8 " "	white "	1.5	medium	2	"	3	good	"
9 " "	crimson "	1.5	"	2	"	3	"	"
10 Crimson "	red "	.5	"	dead				
11 " "	white "		good	"				
12 " "	alsike "		"	2	good	3	good	"
13 Alfalfa	sweet "	1.5-2	medium	2.5	"	4	"	"
14 Sweet clover	Alfalfa	2.	"	2.5	"	4-4.5	"	"
15 Cow pea	soy bean	2-8	good	3-8	medium	3-9	poor	4 small
16 " "	garden bean	2-7	"	3-8	"	4-9	"	none
17 " "	Apios	4-8	"	4-8	"	4-9	"	"
18 Soy bean	cow pea		"	6-9	good	6-9	medium	"
19 " "	garden bean		"	6-8	"	6-9	good	"
20 " "	Apios		"	4	medium	6	"	"

Table VI, continued.

Plant	Inoculated with Bacteria.	Nov.29		Dec.5		Jan.28		Tubercles.
		In. high	color	In. high	color	In. high	color	
21 Garden bean	Cow pea	5-9	medium	6-10	medium	6-10	poor	none
22 " "	soy bean	5-9	"	7-10	"		"	"
23 " "	Apios	5-8	"	6-10	"	6-10	"	"
24 " pea	vetch		"	2-7	"	dead		"
25 " "	sweet pea		"	6-10	"	12	good	numerous
26 " "	partridge pea		"	6-10	"	dead		
27 Vetch	garden "		"	6-10	medium	12	good	numerous
28 "	sweet "		"	6-9	good	12	"	"
29 "	partridge "		"	6-8	medium	8	poor	none
30 Sweet pea	garden "	4-7	"	6-9	"	11	good	numerous
31 " "	vetch		"	4'	dead			none
32 " "	partridge pea	5-7	"	6-9	medium	dead		none
33 Canada F. pea	garden "	3-7	"	4-9	"	30	good	numerous
34 " " "	vetch		"	10	"	12-36	"	"
35 " " "	sweet pea	4	"	5-5	"	16	"	"
36 " " "	partridge pea		good	2-5	"	dead		none
37# Cow pea	" "	3-8	"	3-9	"	4-9	poor	"

From replanting (See No.10)

Table VII.

First Series--Closed Pots--N Analyses.

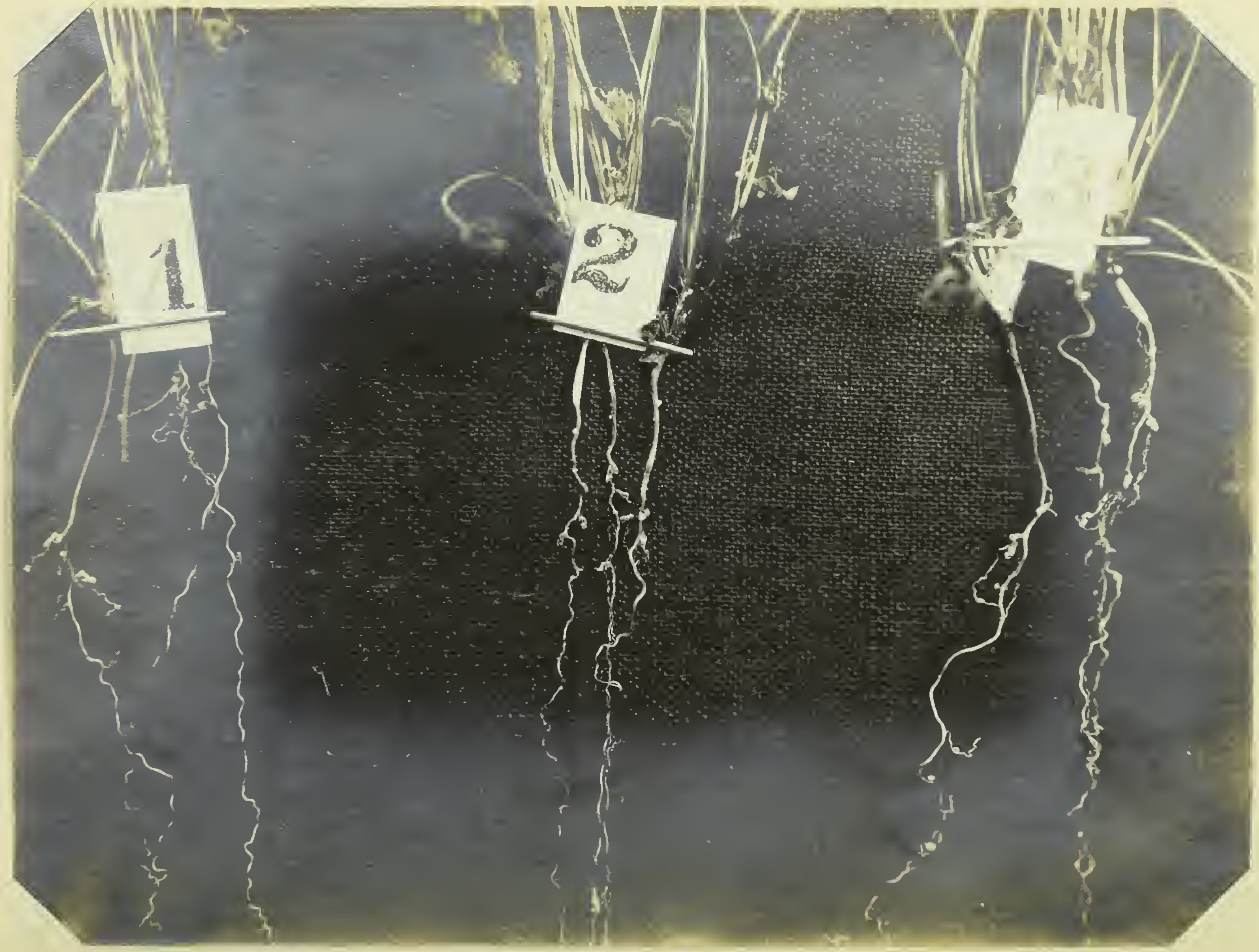
Plant	Bacterium	Tubercles	No. of plants	Mgs. N in plants	Mgs. N in seed	Mgs. N fixed	Mgs. dry matter	%N in dry M
Red clover	white clover	numerous	10	4.41	.957	3.453	121.7	3.62
Red clover	alsike clover	"	10	3.11	.957	2.153	134.4	2.31
Red clover	crimson clover	"	10	3.56	.957	2.603	121.2	2.93
White clover	red clover	"	10	2.80	.354	2.446	90.8	3.08
White clover	alsike clover	"	10	3.43	.354	3.076	104.4	3.28
White clover	crimson clover	"	10	2.58	.354	2.226	87.0	2.96
Alsike clover	red clover	"	10	2.00	.390	1.610	64.52	3.10
Alsike clover	white clover	"	10	2.115	.390	1.720	71.56	2.95
Alsike clover	crimson clover	"	10	1.95	.390	1.560	63.40	3.07
Crimson clover	red clover							
Crimson clover	white clover							
Crimson clover	alsike clover	numerous	10	79.70	2.23	77.570	1615.	4.94
Alfalfa	sweet clover	"	10		1.3		145.7	
Sweet clover	alfalfa	"	10	4.65	1.17	4.53	161.0	2.88
Cow pea	soy bean	4 small	1	9.16	9.11	.066	236.1	3.88
Cow pea	garden bean	none	1	10.24	9.11	1.1	238.7	4.29

Table VII. continued.

Plant	Bacterium	Tubercles	No. of plants	Mgs. N in plants	Mgs. N in seed	Mgs. N fixed	Mgs. dry matter	% N in dry M.
Cow pea	Apios	none	1	9.25	9.11	.15	249.0	3.71
Soy bean	cow pea	"	1	43.35	9.71	33.64	850.	5.10
Soy bean	garden bean	"	1	45.08	9.71	35.37	883.	5.10
Soy bean	Apios	"	1	41.23	9.71	31.52	691.	5.90
Garden bean	cow pea	"	1	8.71	13.76	-5.05	184.6	4.71
Garden bean	soy bean	"	1	7.75	13.76	-6.01	158.0	4.90
Garden bean	Apios	"	1	8.86	13.76	-4.90	198.0	4.47
Garden pea	vetch	died	1	8.64	12.31	-3.67	133.7	6.46
Garden pea	sweet pea	numerous	1	18.35	12.31	6.04	342.0	5.36
Garden pea	partridge pea	none	1	17.29	12.31	4.98	358.0	4.82
Vetch	garden pea	numerous	1	4.61	1.19	3.42	91.5	5.04
Vetch	sweet pea	"	1	4.92	1.19	3.73	125.	4.00
Vetch	partridge pea	none	1	2.02	1.19	.83	127.5	1.58
Sweet pea	garden pea	numerous	1	12.76	2.14	10.62	305.2	4.18
Sweet pea	vetch	none	1					
Sweet pea	partridge pea	"	1					
Canada pea	F. garden pea	numerous	1	7.36	3.48	3.88	208.6	3.52

Table VII, continued.

Plant	Bacterium	Tubercles	No.of plants	Mgs.N in plants	Mgs.N in seed	Mgs.N fixed	Mgs.dry matter	%N in dry M.
Canada F. pea	vetch	numerous	1	26.06	3.48	23.58	724.0	3.60
Canada F. pea	Sweet pea	"	1	15.96	3.48	12.48	453.0	3.52
Canada F. pea	partridge pea	none	1		3.48		114.0	
Cow pea	partridge pea	"	1	9.08	9.10	-.03	215.5	4.25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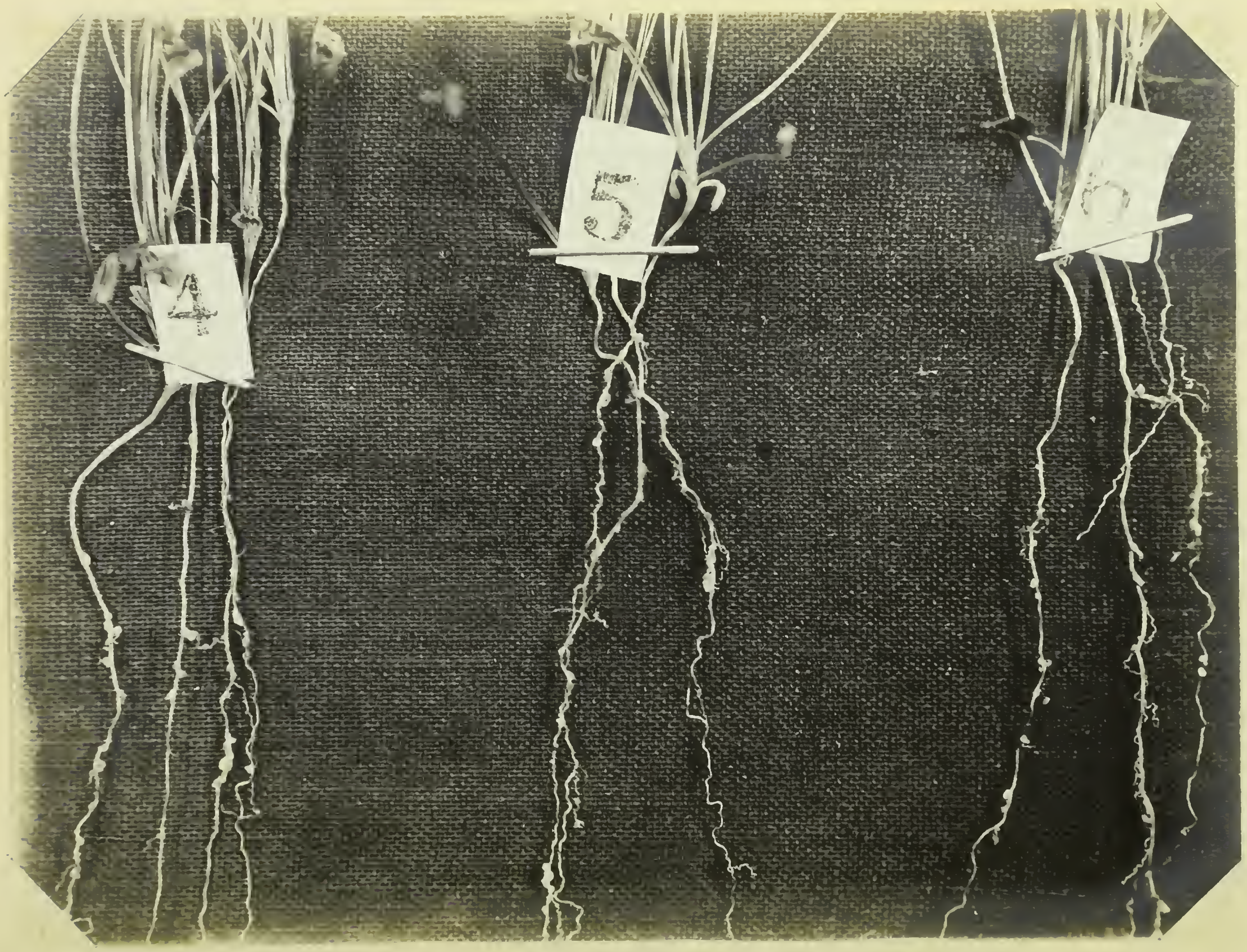
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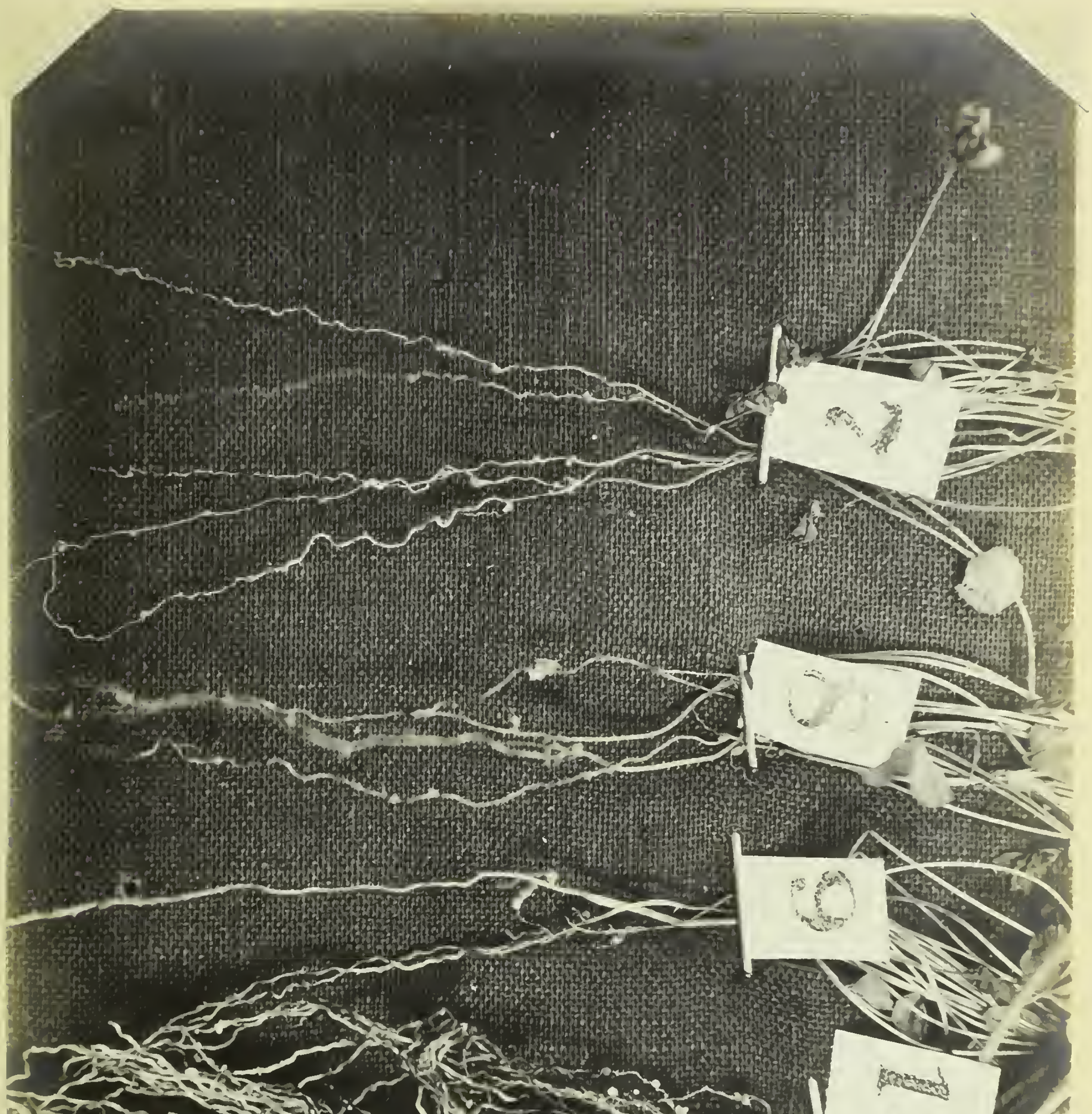
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The above tables show positive infection and fixation in every case in group I, except in the last two where the plants died suddenly, (probably due to excessive heat). Otherwise all the plants did well under the conditions and there seems to be no great advantage of one bacterium over another. White clover bacteria seemed to give the best results on red clover and crimson clover seemed to be not quite so well adapted to its hosts as did the other three forms. Crimson clover inoculated with alsike clover showed remarkable fixation. This is largely explained by the fact that all the plants died but one which had plenty of room for growth.

In group II composed of alfalfa and sweet clover both plants were well infected and grew very well. Unfortunately the nitrogen determination was lost for the alfalfa; but the other data show that they produce good results when crossed.

In group III we found no tubercles developed except a very few with soy bean on cow pea. In all inoculations made upon cow pea and garden bean there was little or no nitrogen fixation and they both had a poor color when washed out. The soy bean, on the other hand showed a wonderful fixation in every case and had a good color throughout. The peculiar fact to note here is that there were no tubercles. This can only be explained by the fact that they were internally infected or that they had unusual feeding powers which the other plants did not have.

Group IV. gave all possible combinations except with Cassia. Garden pea with vetch, and sweet pea with vetch both died and the test is therefore not complete. Good growths and considerable fixation of nitrogen was obtained in all the other pots, thus showing that, garden pea, sweet pea, vetch and Canada field pea bacteria are

all interchangable.

A peculiar fact shown by table VII is the exceedingly high percentages of nitrogen in nearly all of the plants. This is probably due to the conditions under which the plants were grown. The preceding photographs show the development of tubercles upon representative plants of each kind of plants infected. The numbers in the photographs corrospond to the ones in the table.

In order to get a test of all kinds of our legume bacteria on each species of plant, we decided to run a series of open jars and thus shorten the work with the closed jars. We felt quite confident from our grouping and from other experiments that there would be very little crossing between groups; and knowing that the open jars offered better conditions of plant growth we run this series, inoculating each plant with a culture from each group except the one to which the plant belonged (this being done in the closed pots). In this series we have accepted negative results as final; but fearing that positive results might be due to outside infection we have so far as possible repeated them with individual inoculations. The groups are composed as follows:-

Group I.
- red clover
- white "
- alsike "
- crimson "

Group II.
- alfalfa
- sweet clover

Group III.
- cow pea
- soy bean
- garden bean
- Apios

Group IV.
- garden pea
- vetch
- sweet pea
- Canada field pea
- partridge pea

Table VIII.

Open Pots--(Nov.11, 1904--Jan.28, 1905)

Plant	Bacterium inoc.with	December 5		January 20		Tubercles
		In.high	color	In.high	color	
1.Red clover	group II	1-2	medium	2-3	good	numerous
2. " "	" III	1-2	"	2-3	medium	"
3. " "	" IV	1-2	"	1-2	"	"
4.White "	" II	.5-1	"	.5-1	poor	few
5. " "	" III	.5-1	"	1+	"	"
6. " "	" IV	.5-1	"	.5-1	"	"
7.Alsike "	" II	.5-1	"	3.5-4	good	numerous
8. " "	" III	.5-1	"	1+	poor	few
9. " "	" IV	.5-1	good	3.5-4	good	numerous
10.Crimson"	" II	1-1.5	"		medium	few
11. " "	" III	1-1.5	"	1	poor	one
12. " "	" IV	.5-1	"	1.5	good	few
13.Alfalfa	" I	2-3	"	2-6	"	numerous
14. "	" III	2-3	"	2-6	"	"
15. "	" IV	.5-1	medium	1-2	medium	none
16.Sweet clover	" I	.5-1	good	1-2	"	"
17. " "	" III	.5-1	medium	1-2	"	"
18. " "	" IV	.5-1	"	1-2	"	"
19.Cow pea	" I	2-4	good		poor	"
20. " "	" II	2-4	"	4-5	"	"
21. " "	" IV	2-4	"	6	medium	few

Table VIII. continued.

Plant	Bacterium inoc.with		December 5		January 5		Tubercles
			In.high	color	In.high	color	
22.Soy bean	group	I	replanted		6	medium	none
23. " "	"	II	2-4	medium	6-8	good	"
24. " "	"	IV	2-4	"	7-8	"	"
25.Garden"	"	I	4-6	"	8-9	poor	"
26. " "	"	II	5-6	good	8	"	"
27. " "	"	IV	4-6	"	8-9	"	6 small
28.Garden pea	"	I	2-6	"	4-18	good	numerous
29. " "	"	II	3-5	"	4-12	medium	none
30. " "	"	III	2-5	"	4-10	"	"
31.Vetch	"	I	2-5	"	5-8	poor	"
32. "	"	II	3-4	"	6	"	"
33. "	"	III	3-5	"	6-9	medium	"
34.Sweet pea	"	I	3-5	medium	5-6	"	few
35. " "	"	II	2-5	"	4-7	"	none
36. " "	"	III	2-4	"	6-7	"	"
37.Canada F.pea	"	I	3-6	good	5-9	poor	three
38. " "	"	II	3-5	"	6-18	dying	none
39 " "	"	III	4-6	"	12	"	"

In such a series as the above it was impossible to avoid outside infection, but by selecting, only those plants which checked in their results a great deal of this outside infection was avoided. In the above table inoculations were made in groups so that it can not be told directly what bacterium produced infection, but this was found in the following manner. Red clover was infected by groups II, III, and IV. Upon examining group II we found that alfalfa only was infected by group I. The results show that alfalfa and red clover cross inoculate and that sweet clover and red clover do not. The same follows for all the members of group I. Again red clover produced tubercles when inoculated with group III, but upon examining the inoculations on group III we found that no crosses were made by group I. Such one sided crosses were considered as accidental and the results called negative. In a similar manner we run through the series finding the following to be the probable crosses.

Plant	Bacterium	Plant	Bacterium
Red clover	Alfalfa	Alfalfa	red clover
" "	garden pea	garden pea	" "
" "	sweet pea	sweet pea	" "
White "	alfalfa	alfalfa	white "
" "	garden pea	garden pea	" "
" "	sweet pea	sweet pea	" "
Alsike "	alfalfa	alfalfa	alsike "
" "	garden pea	garden pea	" "
" "	sweet pea	sweet pea	" "
Crimson"	alfalfa	alfalfa	crimson"
" "	garden pea	garden pea	" "
" "	sweet pea	sweet pea	" "

Our later work has shown that we may get a cross one way and not the other, but all such one sided crosses have been very poor. We beleive therefore, that the above crosses represent all possibilities of any importance but not necessarily all possibilities.

In our second series of closed jars we included, those plants which died in the first test, all of group III as run in the first series, and as many as possible of the crosses found in the open jars. We made analyses of those plants as in table VII, but owing to the fact that they were not left as long as the first series they did not show fixations which would make it worth while including the analyses. As a general thing those bearing tubercles contained the most nitrogen but there were exceptions to this. In several cases plants from infected pots were smaller than those from pots not infected. This was especially noticable in the crosses between groups. In the following table it should be observed that color is not a guide to infection except in comparing inoculations on the same plant: for instance soy beans always showed good color while cow peas do not have a good color when well infected.

Table IX.

Second Series--Closed Pots--Feb.18--April, 12, 1905.

Plant	Bacterium	March 13		March 22		April 12		Tubercles
		In. high	color	In. high	color	In. high	color	
1 Crimson clover	red clover	.5	medium	.75	medium	1	medium	numerous
2 Crimson clover	white clover	.5	poor	.5-.75	"	.5-1	"	"
3 Garden pea	vetch	2-4	good	3-5	good	10	good	"
4 Sweet pea	vetch	6	"	10	"	12	"	"
5 Red clover	alfalfa	.5	medium	.25-1	medium	.5-2.5	poor	none
6 Red clover	garden pea	.25	poor	.25-.5	"	.5	"	"
7 Red clover	sweet pea	.5	medium	.25-.75	"	.5-2	"	three
8 White clover	alfalfa	.33	"	.25-.5	poor	.5-1.5	"	none
9 White clover	garden pea	.25	"	.25	"	.25-.5	"	"
10 White clover	sweet pea	.25	"	.25	medium	.25-.5	"	four
11 Alsike clover	alfalfa	1.25	"	.25-.5	"	.5	"	none
12 Alsike clover	garden pea	.25	poor	.25	poor	.25	"	four
13 Alsike clover	sweet pea	.25	medium	.25-.5	medium	.5-1	medium	five
14 Crimson clover	alfalfa	.5	"	1	"	1-2	poor	none
15 Crimson clover	garden pea	.25-.75	good	.5-1	"	.5-1	"	few

Table IX, continued.

Second Series--Closed Pots--Feb.18--April, 12, 1905.

Plant	Bacterium	March 13		March 22		April 12		Tubercles
		In. high	color	In. high	color	In. high	color	
16 Crimson clover	sweet pea	.25-75	good	.25-1	medium	1-2	medium	few
17 Cow pea	soy bean	6	medium	7	"	9	"	few-large
18 Cow pea	garden bean	3-6	"	5-6	"	7	poor	none
19 Cow pea	Cassia	3-8	"	5-8	"	6-11	medium	numerous
20 Cow pea	Apios	3-8	"	5-8	"	6-9	"	none
21 Soy bean	cow pea	4-7	good	5-8	good	7-10	good	several
22 Soy bean	garden bean	7	"	7-8	"	9	"	none
23 Soy bean	Cassia	2-6	"	2-8	"	5-9	"	"
24 Soy bean	Apios	2-5	medium	4-7	medium	8	medium	"
25 Garden bean	cow pea	6-8	poor	6-8	poor	9	poor	"
26 Garden bean	soy bean	4-8	"	5-7	"	7	"	"
27 Soy bean	tubercles	4-5	good	6-8	good	8-9	good	"
28 Alfalfa	red clover	.5-.75	medium	.25-1	poor	.5-1	poor	"
29 Garden pea	red clover	2	"	1	"	dead		
30 Alfalfa	white clover	.5-1	"	.5-1	medium	.5-2	poor	"
31 Sweet pea	white clover	5	good	8	"	10	medium	"

Table IX, continued.

Second Series--Closed Pots--Feb.18--April, 12, 1905.

Plant	Bacterium	March 13		March 22		April 12		Tubercles
		In. high	color	In. high	color	In. high	color	
32 Alfalfa	alsike clover	.5-1	good	.5-1.5	mediu	.5-1	poor	none
33 Garden pea	alsike clover	2-3	mediu	4-5	"	dead		
34 Alfalfa	crimson clover	.5-1	good	.5-2	"	.5-2	medium	none
35 Soy bean	check	5	"	3-4	medium	7	"	"
36 Cow pea	cow pea	2-6	"	3-6	good	5-8	"+	numerous

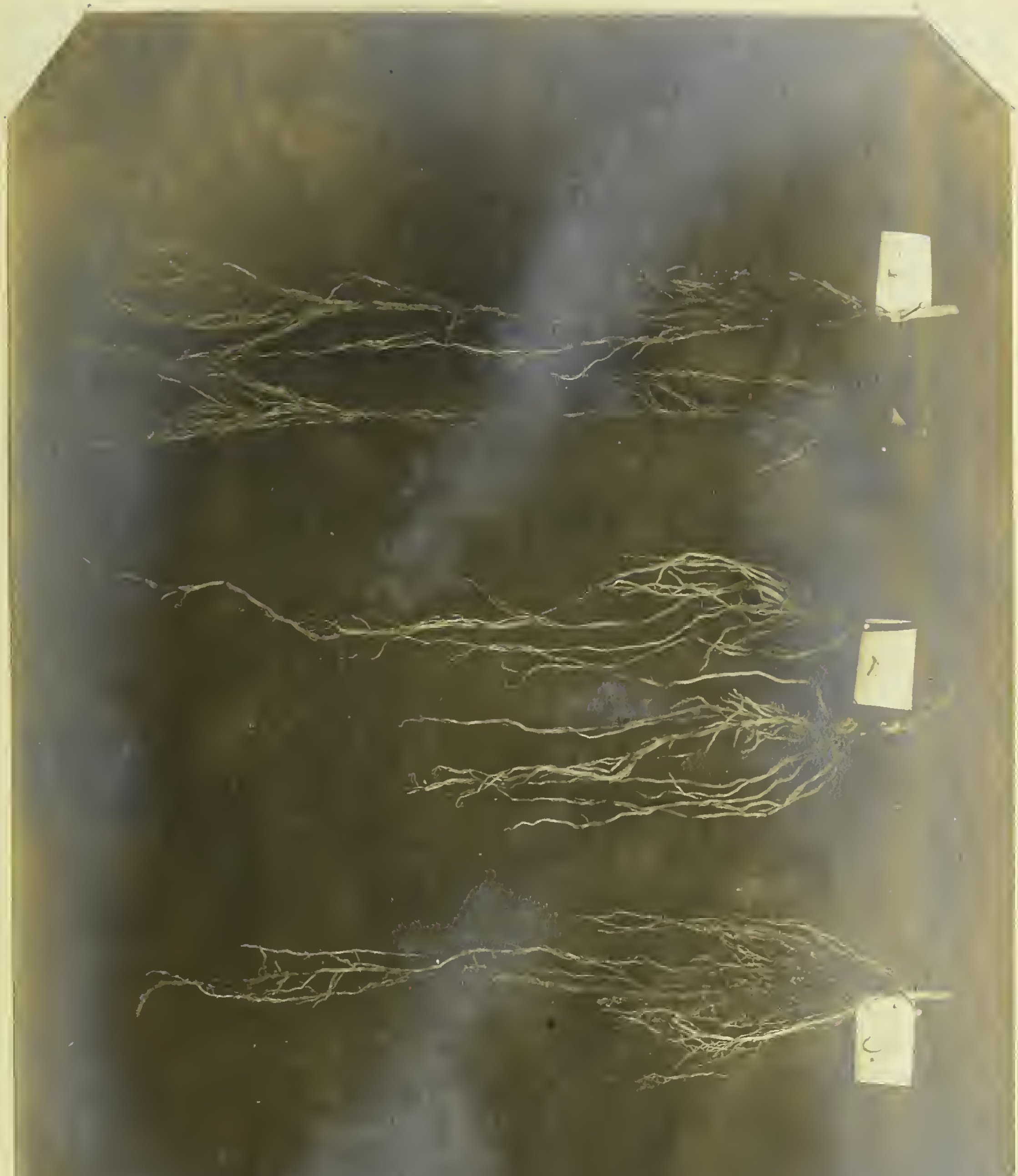
In the above table the first four plants are to replace those which died in the first series. They were all well infected and substantiate the conclusions drawn from the first series. 15--16 and 28--34 inclusive are those crosses which showed positive results in the series of open jars. They no where showed good results; there was either no infection at all or only a few tubercles which did not seem to benefit the plants. These results show that although poor, crosses on widely different plants can be made. And without doubt by cultivating these bacteria for a time they would become adapted to the plant in question.

Cow pea inoculated with soy bean showed better infection than it did in the first series. Cow pea with Cassia showed fair growth and numerous tubercles, while in the first series it showed none. An explanation of this may be found in the fact that in the first series these plants were not inoculated until after they were up while in the latter the cultures were poured over the seed when planted. Soy bean with cow pea bacteria showed numerous tubercles while the first series showed none, although there was good growth in both cases. Soy bean inoculated with sterilized tubercles from soy bean made the best growth of all and showed a considerable fixation of nitrogen but there was no tubercles on the roots. Soy bean uninoculated (35) grew fairly well but showed no fixation of nitrogen. The remainder of this group showed about the same results as in the first series, with the exception of the nitrogen content. This was due to the fact that they were not left as long.

From the results of the first series we were led to beleive that in several instances we had secured nitrogen fixation without the development of tubercles.

This was shown by the analyses of the soy bean plants and by the fact that many of the largest alfalfa plants in a jar bore no tubercles while the smaller ones in the same jar did bear tubercles. In the second series the results were much the same with the exception of the nitrogen fixation which is explained above. After washing out the soy bean plants, we examined sections of the roots with the microscope and found in every case, except the check, what seemed to be bacteria; but these objects were not nearly so numerous as the bacteria found in the tubercle. In several cases we succeeded in staining a few branched objects which greatly resembled bacteria. We sterilized portions of these roots and made inoculations as from a tubercle. In from two to three days about one third of the inoculated tubes developed small transparent colonies which seemed to be identical with the legume bacterial growth. The check plant gave no cultures. Upon washing out plants we observed that in many cases uninoculated plants had a much coarser root system than the inoculated plants. The soy bean check which is No.2 in the accompaning photograph illustrates this point. Numbers one and three are soy bean inoculated with garden bean and soy bean tubercles respectively. This fact gives further evidence that the latter plants were internally infected as it is a known fact that plants having an abundance of plant food supplied, develop fine root systems. From the above facts we conclude that some legumes (soy bean and alfalfa) do fix free nitrogen as the result of internal infection.

At the time of planting of the second series we introduced into the various jars a few seed from the kind of plant from which the inoculating bacteria had been taken, so as to ascertain the purity of our cultures. In every case these plants grew vigorously



and showed numerous tubercles on washing out. In fact they invariably outstripped the other plants in the same pot even when those plants showed infection. From this it appears that each bacterium produces the best results upon its own host and that growing it in pure culture does not destroy this preference.

Summary.

Free nitrogen is fixed in certain species of legumes and a few other plants, through the agency of bacteria.

These bacteria work in the roots of the plants and there in most instances produce tubercles. The tubercles are filled with bacteria and are the seat of nitrogen fixation.

Nitrogen fixation is possible without tubercle development.

There are several species of bacteria and algae which are able to fix nitrogen in the soil without the aid of higher plants.

Each kind of legume bacteria gives the best result~~s~~ upon its own host~~s~~.

Cultivation on artificial media tends to make crosses easier but does not destroy the preference of the bacteria for their natural host.

The relationships of the bacteria are shown by the forms of the bacteroids.

Some crosses give good fixations and are beneficial to the plant, others produce tubercles but do not materially aid the growth of the plant.

Legume bacteria are all rather closely allied, some more closely than others. Some are so far apart that so far as known crosses cannot be made.